

EXHIBIT M

MICROSCOPY OF INTENTIONALLY OXIDIZED POLYPROPYLENE-BASED MESH MATERIAL

*Stephanie J. Benight, Steven B. MacLean, Mariana B. Garcia, and Jericho L. Moll
Exponent, Inc., Menlo Park, CA*

Abstract

Surgical mesh and sutures made from polymer materials have long been utilized as medical devices. Several polymeric materials have been employed to manufacture these devices, including polypropylene. PROLENE[®] fibers, comprised of polypropylene-based material with added antioxidants, pigment, and processing aids are fiber spun and used in medical sutures and pelvic mesh implants. Claims of *in vivo* degradation of mesh devices, including PROLENE[®], have been investigated by others [1-7]. Surgical mesh is typically surrounded by tissue during and after implantation. Histological dyes such as Hematoxylin and Eosin (H&E) can be used to stain surrounding tissue on explanted devices. Using optical microscopy, we demonstrate that non-implanted, intentionally oxidized, PROLENE[®] fibers do not stain with H&E. The inability of PROLENE[®] to become stained is an important finding as it provides histologists and others a means of delineating between biological material surrounding mesh and the fibers that are used to construct the mesh.

Introduction

Surgical meshes are typically implanted in the body for the support of soft tissues, including abdominal wall defects (hernias), uro-gynecological anatomy, and cardio-thoracic defects [8]. Polymeric mesh materials in particular date back to the 1950s [8]. Due to its decades-long success in the field of hernia management, surgical mesh has also become prevalent in the treatment of urological and gynecological conditions, such as urinary incontinence and pelvic organ prolapse.

PROLENE[®] (Ethicon Inc.) is the tradename of the specific polypropylene-based resin used to manufacture several medical devices including mesh and suture products. In addition to the polypropylene neat resin in its formulation, PROLENE[®] is compounded with additives, including antioxidants and other processing aids. In some instances, the additive package also includes a pigment which changes the color of the compound from clear to blue. In order to construct mesh and suture products, PROLENE[®] is first converted into continuous fiber via the melt spinning process. Once the material has been processed, the PROLENE[®] filaments are knit into meshes or cut to specified lengths for sutures. In this study, the PROLENE[®] meshes used consist of both colorless and

blue fiber filaments. PROLENE[®] material is the basis of the work presented herein and will be referred to as a polypropylene-based material from this point forward.

The use of polypropylene as a surgical mesh material has recently come under scrutiny, with claims of alleged degradation while *in vivo*. Despite these allegations, few, if any, accounts of ruptured mesh have been reported. Most reports claim that mesh degradation occurs as a result of oxidation [3, 6, 9]. Oxidation is typically asserted through observation of a combination of cracking and oxygen-related functional groups associated with the outer surface of the mesh material. Specifically, select scanning electron microscopy (SEM) micrographs have shown a cracked coating surrounding the explanted polypropylene (PP) fibers [3, 6, 10]. In addition, Fourier transform infrared spectroscopy (FTIR) has been employed in an attempt to characterize the cracked outer coating layer. Some authors report the resultant spectra contain absorbance bands at or near 1740 cm⁻¹ and solely ascribe these bands to oxidation of the underlying polypropylene [4, 6, 9]. These reports typically extend these observations to suggest that polypropylene is degraded as a result of implantation in the body.

One important factor that needs to be taken into consideration when analyzing explanted mesh is that these materials are typically surrounded by, if not embedded in, tissue after being removed from the body. Upon explantation, it is not uncommon for mesh surrounded by tissue to be preserved and fixed in formalin and subsequently examined using histological stains to visualize the tissue. Hematoxylin and Eosin (H&E) is one of the most widely used histological stains [11]. The process of tissue staining involves mounting samples to create a stiff matrix around the tissue, then sectioning the mounted sample via microtomy to create thinly sliced cross sections. The cross sections are then adhered to slides and treated with a series of dehydrating washes and stains prior to imaging with microscopy. H&E staining will result in tissue appearing various shades of pink, purple, red or blue, depending on the type of tissue in the sample.

The mechanism for H&E staining of biological tissue consists of simple ionic bonding between molecules of opposite charge: specifically, charge on the H&E dye molecular complex and charge on the biological molecules that comprise the tissue [12]. The hematoxylin dye itself is a mixture of hematoxylin, hematein, aluminum ions, and solvent used in combination with a “mordant” compound (typically aluminum). The resultant

molecule is cationic (positively charged) and seeks to ionically bond with negatively charged molecules [11, 12]. Eosin, used in combination with Hematoxylin, is negatively charged and attracts positively charged molecules. Cationic hematoxylin reacts with negatively charged, basophilic cell components, such as nucleic acids in the nucleus, ribosomes, and acidic mucin. In contrast, Eosin reacts with and stains positively charged biological structures such as cellular membranes, cytoplasm, connective tissue, and extracellular matrix tissue [11, 12]. Furthermore, some amino acids, such as the molecular building blocks of proteins (which are also polymeric), contain a charge. These charged compounds will also form ionic bonds with H&E and appear stained.

In some reports, implants that have allegedly oxidized have been removed from the body and histologically processed using H&E stains [6, 10]. Contrary to the known *chemical* staining mechanism associated with H&E stains, some authors contend that oxidized polypropylene can become stained through *physical* entrapment of the stains [10]. Entrapment sites are reported to include voids and crevices such as cracks, nanopores, and nanocavities that supposedly manifest within the material as a consequence of the alleged oxidation process [10].

Oxidized polypropylene molecules are not expected to ionically bond with H&E stains. Likewise, unadulterated polypropylene molecules are not ionically charged and are not expected to stain with H&E. In addition, other polymer implant materials such as UHMWPE and PMMA have also shown an inability to stain when subjected to H&E stain [13-15].

Herein we evaluated whether or not an intentionally oxidized polypropylene-based material stains with H&E when processed through a typical staining protocol. Our initial hypothesis was that non-oxidized and intentionally oxidized material would not become stained after processing. To validate this hypothesis, the polypropylene-based mesh was intentionally oxidized using two different techniques; a chemical method reported to oxidize PROLENE® [9] and 2) ultraviolet (UV) light exposure [16] (See Materials section below). After subjecting intentionally oxidized samples to a typical embedding, sectioning, and staining protocol, the polypropylene cross sections were imaged using polarized and non-polarized optical microscopy. The efficacy of the H&E staining procedure employed in this study was confirmed by incorporating a positive control (rabbit tissue) into the experimental design. As expected, the rabbit tissue readily stained and was used as a reference sample throughout the study.

Materials

PROLENE®, a polypropylene-based material produced by Ethicon, Inc., is the base material used in this study. As with many commercially available resin compounds, PROLENE® resin is comprised of several raw material ingredients in addition to the base isotactic polypropylene including antioxidants and other processing aids. The pristine PROLENE® mesh (Ethicon TVT Device, Ref. No. 810041B, Lot No. 3661669) used in this study was kept in its original packaging until use.

Sections of PROLENE® mesh were chemically treated according to the protocol published by Guelcher and Dunn [9]. Samples were incubated at 37°C for up to 5 weeks in oxidative media composed of 0.1 M CoCl₂ in 20 wt% H₂O₂. This solution purportedly simulates the oxidative environment created by macrophages in response to a foreign object. The oxidative solution was changed every 2-3 days. Prior to processing, the samples were copiously rinsed in de-ionized water, sonicated overnight at room temperature, air-dried, and assessed for morphological changes using SEM.

Additional samples of PROLENE® mesh were placed inside a Q-Lab QUV Accelerated Weathering Tester and irradiated with 0.98 (W/m²) UV at 60°C for 5 days. As with the meshes exposed to chemical treatment, the samples were assessed for morphological changes using SEM prior to processing. As a control, pristine exemplar mesh samples, which had never been implanted or subjected to oxidation, were also subjected to the staining protocol.

For the QUV oxidized PROLENE® mesh experiments, six (6) pieces (samples) of pristine, out-of-the-box PROLENE® mesh were cut with a razor blade from a TVT device. Each sample, and its corresponding individual fiber segments, was exposed to ultraviolet (UV) light and observed to be cracked by SEM examination. Six additional pieces were cut from the TVT device with a razor blade and chemically treated. Each sample, including the UV treated sample, the chemically treated sample, and the exemplar sample, contained approximately 120 individual fiber segments.

In order to facilitate the microtomy of individual fibers, mesh samples were embedded in both paraffin and resin (Technovit). Once embedded, the samples were sectioned and then stained with Hematoxylin & Eosin. All processing was performed by a commercial histology lab.

For paraffin embedding, samples were dehydrated in reagent alcohol and Xylene substitute using an automated tissue processor, then embedded in Leica EM400 Paraffin wax. Sections of the paraffin blocks (4-6 µm thick) were obtained using a microtome, briefly floated in a 40-45°C water bath, then mounted onto slides. Sections were air-dried for 30 minutes then baked in a 45-50°C oven overnight.

For resin embedding, samples were sequentially dehydrated in reagent alcohol using an automated tissue processor, then embedded in Technovit 7200 resin. The

polymerized resin block was trimmed, cut, and ground to a thickness of approximately 50 μm .

Paraffin and resin-embedded samples were stained with aqueous Eosin and Harris Hematoxylin using an automated stainer.

Each sample treated with UV light and chemical solution was used to make several thin, cut slices of mesh embedded in paraffin or resin prior to being subjected to the staining protocol. Specifically, approximately 22 individual fibers of pristine, out-of-the-box PROLENE[®] went through the staining protocol and were available for microscopic examination. In addition, more than 100 individual fibers that were exposed to UV light were subjected to the staining protocol and preserved on microscopy slides. Furthermore, approximately 22 individual fibers that were subjected to chemical treatment were subjected to staining and were preserved on microscopy slides. Finally, out of all of the individual cross sections of fibers segments examined, none were found to retain histological stain.

Results and Discussion

Samples were exposed to either UV or chemical oxidation for approximately 5 days and 5 weeks, respectively. The morphological effects of the oxidation were evaluated using scanning electron microscopy (SEM). Cracks were visible on the exterior mesh surface of the UV-treated samples, but not on the chemically treated samples as shown in Figures 1 and 2, respectively. The lack of cracking on the outer surface of the chemically treated samples is consistent with the microscopic observations made by previous authors [9]. Although “pitting” and “flaking” in polypropylene meshes has been reported [9], no such features were observed in this study.

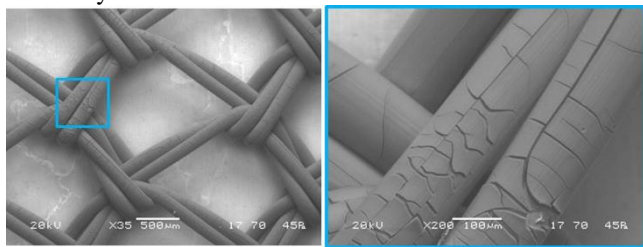


Figure 1. Scanning electron microscope images of UV oxidized mesh.

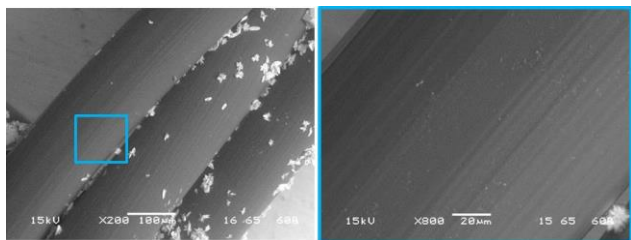


Figure 2. Scanning electron microscope images of mesh that was chemically treated [9].

In the case of the UV treated samples, FTIR spectra exhibited a band at $\sim 1740\text{ cm}^{-1}$, consistent with carbonyl (C=O) functionality that would be associated with polypropylene oxidation given the controlled testing environment (Figure 3). Samples that were chemically treated were also analyzed by FTIR and exhibited a similar oxygen-related band in this same spectral range.

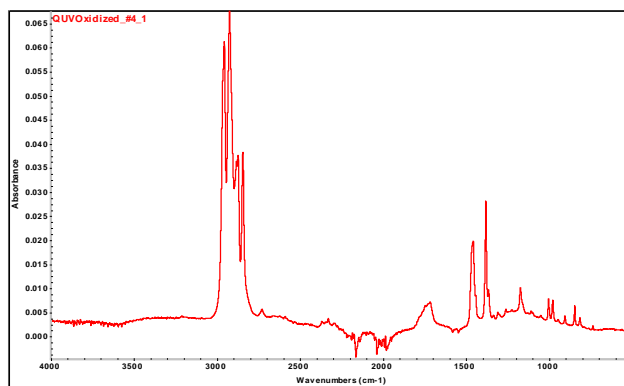


Figure 3. FTIR spectrum of UV treated mesh.

Subsequent to SEM and FTIR characterization, samples were embedded, sectioned, and stained according to the previously outlined protocol in the Materials Section. Evaluation of the effects of stain exposure was performed by optical microscopy of the sectioned samples using polarizers in the typical polarizer/analyzer configuration. As expected for anisotropic structures with fiber symmetry, brightness was highest when the samples were aligned at an angle to the primary polarizing plane (4 locations of maximum intensity) and minimized when aligned parallel to one of the two polarization planes.

The optical microscopy revealed that none of the oxidized mesh samples (both chemical and UV exposed) retained the H&E stain. In contrast, the positive control of proteinaceous rabbit skin tissue readily stained when processed in parallel with the mesh fiber samples, thus validating the staining protocol efficacy. A comparison of the polypropylene-based sample and the stained rabbit tissue is shown in (Figure 4).

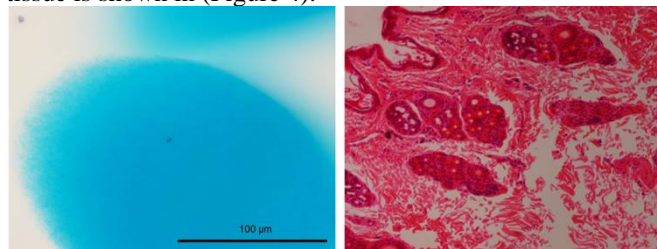


Figure 4. Stark contrast of rabbit tissue that has been treated with stain (right) and polypropylene-based

exemplar material that has also been treated with stain (left).

Given the extensive cracking in the UV exposed specimens observed under SEM, ample opportunity for stain trapping existed from a morphological perspective. Despite multiple observations using high and low magnifications, polarized and non-polarized light, no evidence of the stain being trapped, captured, or otherwise bound within the cracks of the intentionally damaged mesh was observed (Figure 5).

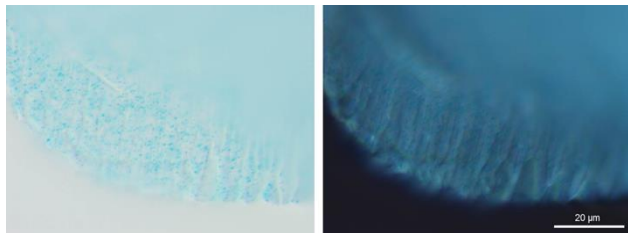


Figure 5. UV treated mesh exhibiting several cracks but no evidence of H&E stain. Image on the left was acquired in absence of polarization, the image on the right was taken with polarization.

As a further control experiment, exemplar mesh samples with no prior exposure to UV light or chemical oxidation were also subjected to the same staining protocol. No evidence of the H&E stain bonding to the polypropylene was observed, confirming that H&E is not effective in staining non-proteinaceous or non-ionic materials (Figure 6).



Figure 6. Pristine (exemplar) mesh embedded in paraffin (left) and resin (right), stained with Hematoxylin & Eosin.

Conclusions

Polypropylene-based surgical mesh was shown not to stain with H&E histological dyes, even after aggressive intentional oxidation with either UV light or chemical treatment.

Conflict of Interest Disclosure

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References

1. A. Clavé, H. Yahi, J.-C. Hammou, S. Montanari, et al., *Int. Urogynecology J.*, **21**(3), 261–270 (2010).
2. R. De Tayrac, V. Letouzey, *Int. Urogynecology J.*, **22**(7), 775–780 (2011).
3. C. R. Costello, S. L. Bachman, B. J. Ramshaw, S. A. Grant, *J. Biomed. Mater. Res. B Appl. Biomater.*, **83B**(1), 44–49 (2007).
4. M. J. Cozad, D. A. Grant, S. L. Bachman, D. N. Grant, et al., *J. Biomed. Mater. Res. B Appl. Biomater.*, 455–462 (2010).
5. T. C. Liebert, R. P. Chartoff, S. L. Cosgrove, R. S. McCuskey, *J. Biomed. Mater. Res.*, **10**(6), 939–951 (1976).
6. C. Mary, Y. Marois, M. W. King, G. Laroche, et al., *ASAIO J.*, **44**(3), 199–206 (1998).
7. A. J. Wood, M. J. Cozad, D. A. Grant, A. M. Ostdiek, et al., *J. Mater. Sci. Mater. Med.*, **24**(4), 1113–1122 (2013).
8. A. S. Pandit, J. A. Henry, *Technol. Health Care*, **12**(1), 51–65 (2004).
9. S. A. Guelcher, R. F. Dunn, *Int Urogynecol J*, **26**(Suppl 1), S55–S56 (2015).
10. V. V. Iakovlev, S. A. Guelcher, R. J. Bendavid, *Biomed. Mater. Res. B Appl. Biomater.*, 000–000 (2015).
11. T. Veuthey, G. Herrera, and V. I. Doderio, *Front Biosci* (Landmark edition), **19**, 91 (2014).
12. Myers, R, *The Basic Chemistry of Hematoxylin*. Available from: <http://www.leicabiosystems.com/pathologyleaders/the-basic-chemistry-of-hematoxylin/> (2011).
13. W. Barbour, S. Saika, T. Miyamoto, and Y. Ohnishi, *Ophthalmic Res*, **37**, 255–261 (2005).
14. R. M. Baxter, A. Ianuzzi, T. A. Freeman, S. M. Kurtz, M. J. Steinbeck, *J Biomed Mater Res A*, **95** (1) 68–78 (2010).
15. M. S. Kung, J. Markantonis, S. D. Nelson, and P. Campbell, *Lubricants*, **3**, 394–412, (2015).
16. Experiments conducted in line with ASTM G154, Standard Practice for Operating Fluorescent Light Apparatus for UV Exposure of Nonmetallic Materials.